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LRIG1 LOCALIZATION WITHIN THE COLONIC CRYPT OF *Lrig3* DOUBLE NULL MICE

by

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A THESIS

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Annie Powell

Leucine rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is a transmembrane protein that negatively regulates intestinal epidermal growth factor receptors (EGFRs) on the cell surface. This evidence suggests that LRIG1 suppresses cell growth and decreases the rate of cellular division within the intestine. Indeed, colonic tumorigenesis is observed in *Lrig1*$^{-/-}$ mice, definitively proving that LRIG1 is an intestinal tumor suppressor protein necessary for tissue homeostasis. Leucine rich repeats and immunoglobulin-like domains protein 3 (LRIG3) localization and functionality are fairly unknown. However, LRIG3 shares many morphological characteristics of LRIG1, which suggests LRIG3 may have similar localization and functionality as LRIG1. Indeed, current studies support this hypothesis, leading this paper to suggest LRIG3 may act as a redundant pathway for tumor suppression for LRIG1 within the colon. As such, this study analyzes shifts in colonic crypt LRIG1 localization in the absence of *Lrig3*. An upward trend in LRIG1-expressing cells is observed in the colonic crypts of *Lrig3*$^{-/-}$ mice, suggesting that *Lrig3* product may

restrict the upward migration of LRIG1. A concurrent decrease in Ki67/LRIG1 co-positive cells within these colonic crypts supports the observation that LRIG1 levels increase in absence of *Lrig3* product. Therefore, this study suggests that LRIG3 may be localized above LRIG1 within the colonic crypt, and that absence of LRIG3 causes LRIG1 to extend its localization, possibly in order to act as a tumor suppressor in cells that would normally possess the redundant tumor suppressor LRIG3.

Acknowledgements

I would like to thank Dr. Powell, Professor Mossberg, and Dr. Stankunas for helping me to investigate and properly express both the context and results of my research involving the cellular composition of the colonic crypt. It is with sincerest gratitude that I was privileged and honored to be guided by such exemplary scholars through this strenuous yet rewarding process. Heartfelt thanks also goes out to the members of the Powell lab for their knowledge and cheer; they encouraged and worked with me to ensure that I felt welcomed and supported. Lastly, I want to thank my family for always helping me on my path in life and for providing me motivation to succeed in both completing this thesis and in fulfilling my double major requirements.

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Introduction

Background: The colon

In order to convert the chemical energy contained within food into usable energy for powering the body, many organisms require a digestive system specialized in both breaking down food into nutrients and in eliminating food waste that is not taken up by the body. For the second part of this process, vertebrates possess a long, tubular organ called the large intestine, of which the cecum, colon, rectum, and anal canal are all considered a part of and which Figure 1 displays. The location of focus for this paper is the composite colon of the large intestine.

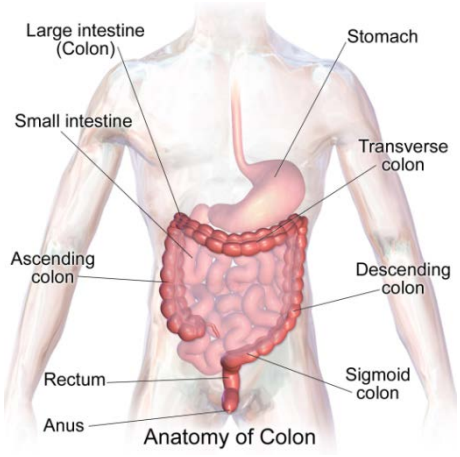

Figure 1: Anatomy of the large intestine.

The large intestine contains the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anus. This organ functions primarily to absorb water into the bloodstream and to condense waste material into feces for defecation. (Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". *WikiJournal of Medicine* **1**(2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436.)

Chyme[1] that enters the large intestine from the small intestine will contain few nutrients and only around 10% of its original water content due to the absorptive nature of the preceding gastrointestinal tract. Even so, the colon will absorb most of the remaining water from the chyme as well as any remaining nutrients. Consequently, the indigestible parts of the ingested food, such as amylose and dietary fiber, will constitute the majority of the material that the large intestine processes. Bacteria living within the colon are able to break down some of the undigested carbohydrates for their own sustenance, producing acetate, propionate, and butyrate as waste products (Miller and Wolin et al., 1996). These short chain fatty acids are taken up by cells lining the colon as a means of nourishment. Furthermore, colonic bacteria produce important nutrients such as vitamin K, vitamin B12, thiamine, and riboflavin that are absorbed by the colon for distribution and use in the host body. As the chyme mixes with mucus and bacteria, it becomes a watery feces that solidifies the farther it travels down the colon, eventually forming a solid stool ready for elimination.

The colonic crypt

Many tissues within the body undergo a constant process of regeneration in order to replace the millions of cells that are lost either through natural cell death or injury (Wath et al., 2013). This process must be tightly controlled in order to prevent malignancies from arising as a result of unregulated cell growth, such as when a tumor forms. The intestinal lumen[2] is one of the fastest sites of cellular self-renewal, with the

[1] Cyme – the semi-fluid mass of gastric juices and partly digested food that leaves the stomach and passes through the small intestine before entering the large intestine.
[2] Lumen – the inside space of a tubular structure; in this case, the intestinal lumen is the hollow inside of the pipe-like small and large intestine.

entire intestinal epithelium[3] replaced every 3-5 days in humans due to the approximately 10^{11} epithelial cells that are sloughed off daily by the passage of chyme (Wath et al., 2013). This monolayer of rapidly regenerating epithelial cells functions to absorb water and nutrients into the bloodstream, while at the same time forming a protective barrier against pathogens[4] and harmful substances.

These new, functional epithelial cells are produced from highly proliferative stem cells[5] found at the base of small, tubular pits called crypts that line the entire intestinal tract and number in the millions (Figure 2).

[3] Epithelial – the thin tissue lining the outside of the body and the inside of hollow structures within the body; the intestinal lumen is lined by this tissue.
[4] Pathogens – bacteria, viruses, or other microorganisms that can cause disease.
[5] Stem cell – a cell with the ability to indefinitely produce more cells of the same type, and from which other, specialized cells can arise via the process of differentiation.

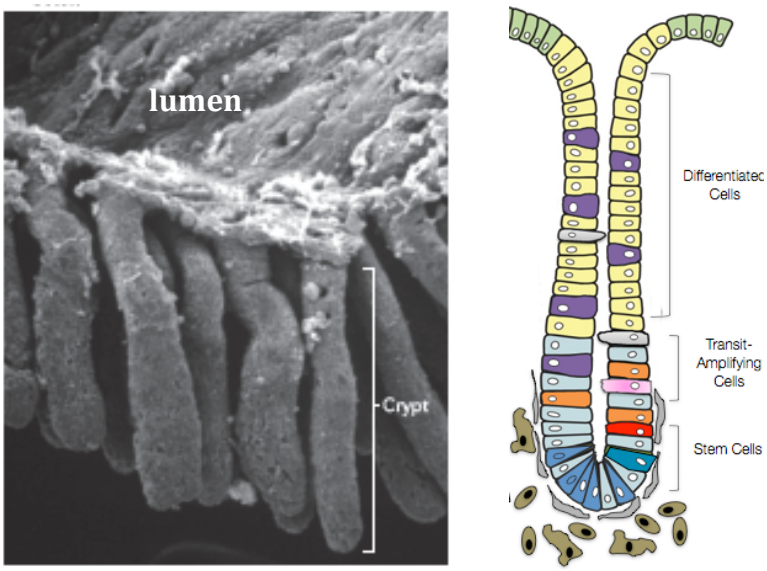

Figure 2: Intestinal crypt cell morphology.

The lining of the colon is punctured by invaginations that form pockets of tissue called crypts (left) (Barker, 2014). Crypts possess a base of self-renewing stem cells above which differentiated daughter cells maintain the morphology and biochemical environment of the crypt (right) (Powell Lab, 2017).

The very bottom, or most basal, cell of the colonic crypt is designated '0'; the next cell up on either side of the crypt is labeled as '+1', the cell after that '+2', and so forth. Referring to a cell within the crypt will therefore done with a '+position' label. The greater the +position number, the more lumenal (closer to the lumen of the colon) the cell is. Specific, specialized cells are often found in only certain +positions within the crypt.

Recent Ki67[6] immunostains[7] suggest that there is a relatively sharp distinction between the proliferative, primitive stem cells at the base of the crypt and the mature, fully differentiated[8] colonocytes further up the crypt wall (Leedham et al., 2012). The direct progeny of these stem cells, termed transit-amplifying (TA) cells, are semi-

[6] Ki67 – a nuclear protein that is used in immunohistochemistry as a marker for cell proliferation; it is an excellent marker for determining the growth fraction of a cell population.
[7] Immunostain – an antibody-based method of detecting a specific protein in a sample.
[8] Differentiated – when a cell changes from one cell type to another, usually more specialized, type.

differentiated and proliferate for several rounds of division before withdrawing from the cell cycle and migrating up the crypt wall, after which they differentiate into mature cells (Potten and Loeffler, 1990). Altogether, the colonic crypt is divided up into three fairly distinct sections: a base of stem cells, TA cells just above the stem cells, and fully differentiated and mature cells that make up the rest of the crypt wall.

As such, cell production, differentiation, migration, and turnover[9] of this tissue is tightly regulated by mechanisms that ensure the quality and proper rate of epithelial cell maturation and detachment. Dysregulation or malfunction of any of these processes is strongly correlated with the development of gastrointestinal disease and the formation of invasive tumors (Radtke and Clevers et al., 2005) (Boman et al., 2008).

Background: Leucine rich repeats and immunoglobulin-like domains

Leucine rich repeats and immunoglobulin-like domains (LRIG[10]) is an evolutionarily conserved[11], single-pass transmembrane[12] protein family that modulates several signaling pathways within the cell. Specifically, the LRIG family has been implicated as essential regulators of tyrosine and serine/threonine kinase[13] growth factor receptors (Simion et al., 2014). In vertebrates, there are three protein members – LRIG1, LRIG2, and LRIG3 – that have been speculated to be mechanistically similar due to their highly related morphology. This morphology consists of leucine-rich

[9] Cell turnover – the process by which new cells travel from one position to another before being shed; this process prevents the buildup of dead cells.
[10] Protein products of genes are fully capitalized, while names of genes are italicized.
[11] Genes (and their products) that are evolutionarily conserved are usually unique and essential for the organism's health.
[12] Single-pass transmembrane (protein) – a protein with one part of itself that is extracellular and one part that is intracellular; these two parts are connected by a domain that rests within the lipid bilayer of the cell.
[13] Kinase – a family of enzymes that catalyze the transfer of a phosphoryl group from a nucleotide triphosphate donor (such as ATP) to an acceptor molecule. This phosphorylation can control enzyme activity, subcellular localization, interactions between molecules, and signal transduction cascades.

repeats, three immunoglobulin-like domains, a transmembrane domain, and a large cytoplasmic tail[14] (Figure 3).

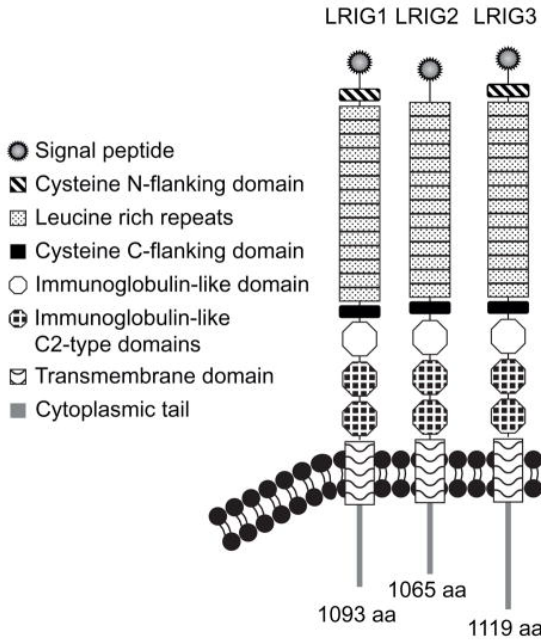

Figure 3: Leucine rich repeats and immunoglobulin-like domains (LRIG).

This transmembrane protein has both an extracellular domain and an intracellular domain, linked by a transmembrane domain that resides in the lipid bilayer of the cell (Simion et al., 2014).

Healthy human tissue broadly expresses LRIGs; indeed, at least twenty-six distinct tissue types have been found to contain detectable amounts of LRIG transcripts (Guo et al., 2004).

While similar homology is shared in the extracellular domains and also in the juxtamembrane portion of their cytoplasmic domains, LRIGs diverge significantly from each other in the remainder of their cytoplasmic domain. As this portion of the protein is responsible for the direct regulation of its host cell, unique functions may be seen between the different LRIGs (Abraira et al., 2010).

[14] Cytoplasmic tail – a part of a protein that extends into the interior (cytoplasmic) portion of a cell.

LRIG1

Intestinal LRIG1 is able to control cell growth via negative regulation of epidermal growth factor receptor (EGFR) signaling, also known as ErbB receptors (Powell et al., 2012). EGFRs are transmembrane proteins that bind extracellular growth factors; as a result of binding, the receptor initiates a cascade of biochemical responses within the cell, the final outcome being one of cell growth promotion. Because LRIG1 functions to restrict the activity of EGFR – and as a result repress cell growth – it is considered a tumor suppressor, or gene product that helps to prevent the transformation of a healthy cell into a cancerous cell. Figure 4 summarizes both the normal mechanistic pathways of EGFR, as well as how LRIG1 inhibits these mechanisms.

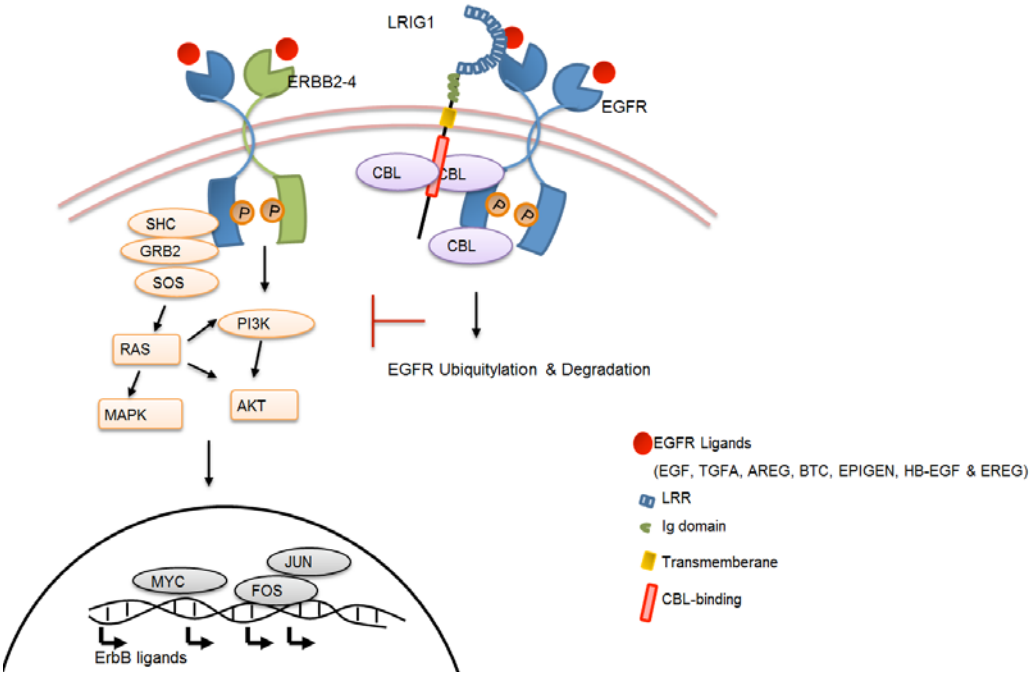

Figure 4: LRIG1 inhibition of an epidermal growth factor receptor (EGFR) signaling cascade.

Ecto-domain interaction of LRIG1 is thought to initiate EGFR ubiquitination and degradation, leading to decreased growth promotion of the cell (Wang et al., 2013).

Evidence supporting LRIG1's functionality as a tumor suppressor resulted in several studies that analyzed the relationship between LRIG1 and cancer. It was found that most, but not all, cancer types examined had decreased levels of LRIG1 relative to normal tissue, with greater decreases in expression associated with worse patient prognoses (Rouam et al., 2010). This suggests that LRIG1 dysregulation may contribute to tumor formation and/or severity.

Lrig1 mRNA lineage tracing[15] revealed cell labeling of the crypt base that, over time, populated the entire crypt; this confirmed that intestinal stem cells are marked by *Lrig1*, and that the LRIG1 protein may be a stem cell marker (Figure 5).

[15] Lineage tracing – identifies all progeny of a cell; in this case, the progeny of colonic crypt stem cells.

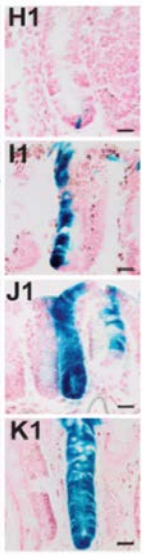

Figure 5: Lineage tracing of *Lrig1*-positive cells in the colonic crypt.

β-galactosidase activity (blue) reflected *Lrig1* mRNA expression over time. Initially labeled crypts contained at least one or two β-gal-positive cells, virtually all of which were located at the base of the crypt (H1). Seven days later, β-gal staining was more widespread, indicating proliferation of initially tagged cells that had produced β-gal-positive progeny (I1). Fully labeled crypts were observed at 14 days post initial staining (J1). Finally, 10% of crypts were entirely lineage traced at 90 days after the initial injection (K1) (Powell et al., 2012).

Additionally, LRIG1-positive stem cells were found to have lower proliferation character and increased cell cycle repression relative to Lrg5 (leucine rich repeat containing G-protein coupled receptor-5)-positive stem cells. This suggests that LRIG1 expressing cells may be relatively quiescent[16]. However, irradiation injury to the crypts resulted in increased LRIG1-positive stem cell proliferation and repopulation (Powell et al., 2014). This suggests that LRIG1 can be repressed to allow for increased cell division when such growth is needed.

[16] Quiescent – the cell is in a state of inactivity or dormancy in regards to cell division.

LRIG3

While the morphology of LRIG3 is very similar to that of LRIG1, recent studies suggest LRIG3and LRIG1 functionally oppose each other. Specifically, LRIG3 has been shown to increase expression of ErbBs 1-4, while LRIG1 promotes LRIG3's proteolytic degradation[17] (Rafidi et al., 2013). This opposition isn't uncommon between members of a protein family involved in cellular signaling, and may have evolved as a means of autoregulation[18]. Moreover, these actions suggest LRIG3 might functionally oppose LRIG1's tumor suppressing abilities, possibly as part of a greater mechanism used to balance cell growth.

However, other studies suggest that LRIG3 might actually function as a redundant[19] tumor suppressor for LRIG1. In one such study, it was discovered that *Lrig3* double null[20] (*Lrig3*$^{-/-}$) mice suffered from deformities of the inner ear during morphogenesis[21] while both *Lrig1*$^{-/-}$ and *Lrig2*$^{-/-}$ mice had morphologically normal inner ear development (del Rio et al., 2013). However, in *Lrig1*/*Lrig3* double null mice[22] there was a worsening of the inner ear phenotype in sites of LRIG1/LRIG3 co-expression. This suggests that the inner ear possesses a level of functional redundancy between LRIG1 and LRIG3, such that the absence of one protein is functionally covered by the presence of the other protein. Furthermore, it is important to note that

[17] Proteolytic degradation – the breakdown of a protein into its component parts such that it is cannot function.
[18] Autoregulation – the process of adjusting (or mitigating) a biological system's response to stimuli; in the case of LRIG1 and LRIG3, cell growth repression (via LRIG1's degradation of EGFRs) is mitigated by LRIG3 (which promotes cell growth via upregulation of EGFRs).
[19] Redundant (gene) – one gene with a given biochemical function is support obliquely by a different gene that preforms the same function; if one gene is damaged or unable to perform normally, its function is taken up by the second gene and so prevents a total collapse of that biochemical function.
[20] Double null ($^{-/-}$) – in which an organism completely lacks its referenced gene's product(s); in this paper, both alleles of the *Lrig3* gene are absent and no *Lrig3* gene product is made anywhere within the organism.
[21] Morphogenesis – the development of the body of an organism.
[22] *Lrig1*$^{-/-}$ and *Lrig3*$^{-/-}$

Lrig1/*Lrig3* double null mice displayed multiple defects in multiple tissues, and either died *in utero*[23] or at birth. These observations support the theory that multiple tissues employ functional redundancy between *Lrig1* and *Lrig3*, and that the absence of both results in widespread homeostatic[24] dysregulation. Therefore, because LRIG1 generally functions as a tumor suppressor via cell growth depression, there is evidence to suggest that LRIG3 may also possess similar abilities.

Unpublished data offers evidence of *Lrig3* mRNA localization within the colonic crypt (Figure 6).

[23] *In utero* – within the womb.
[24] Homeostasis – the state of stable equilibrium established from dynamic processes; physiological homeostasis is vital for an organism's health.

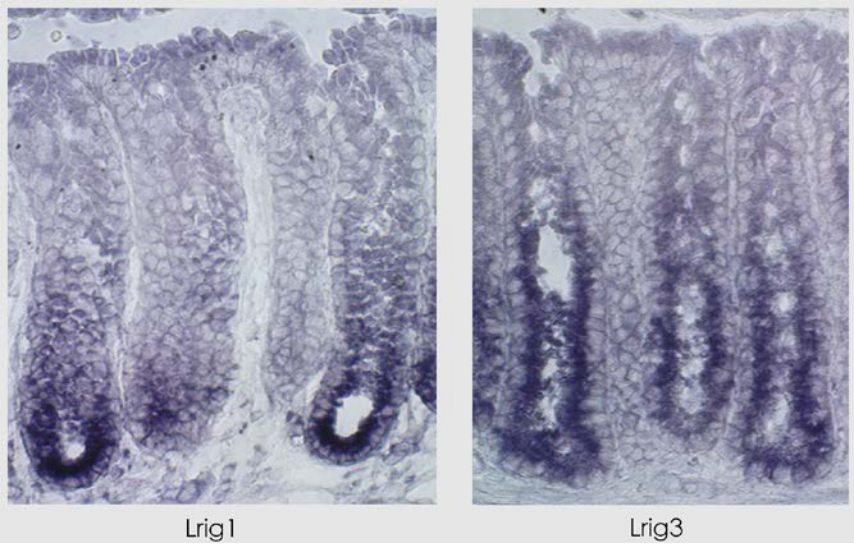

Figure 6: Comparison of *Lrig1* and *Lrig3* mRNA localization within the colonic crypt via *in situ* hybridization[25].

Lrig1 mRNA is found to be concentrated at the base of the colonic crypt in mice, while *Lrig3* mRNA appears to be localized from the base of the crypt (overlapping *Lrig1* mRNA expression) up to approximately 2/3 the height of the crypt (unpublished data, Coffey Laboratory, Vanderbilt University).

This evidence places *Lrig1* mRNA within the base of the colonic crypt, while *Lrig3* mRNA is found to both overlap *Lrig1* mRNA at the base of the crypt as well as extend at least halfway up the crypt wall. This suggests that *Lrig1* and *Lrig3* transcripts interact at the very least within the cells located at the bottom of the colonic crypt. Moreover, if LRIG3 acts as a redundant tumor suppressor to LRIG1, we can see here that *Lrig3* transcripts are located within a useful position to do so as it covers where LRIG1 is normally localized.

Knowledge of LRIG3 expression in cancer is uncertain, with available evidence only suggesting that this protein is tissue- and subcellular localization-dependent in regards to cancer prognoses. One study correlated increased LRIG3-expressing cells in

[25] *In situ* hybridization – labeled complementary DNA, RNA, or nucleic acid probes are used to determine the location of specific DNA or RNA sequences in a portion of tissue; in this case, *Lrig3* transcripts were localized within the colonic crypts.

cervical adenocarcinoma with prolonged survival (Muller et al). Another study found that LRIG3 was an independent prognostic factor in astrocytomas, with elevated levels of LRIG3 linked to a better outcome (Guo et al., 2006). These data are similar to the data obtained from studies of LRIG1 expression in cancers, and further support the idea that LRIG3 may operate as a tumor suppressor, possibly as a redundant pathway for LRIG1 function.

Composite evidence of LRIG3 function

The composite evidence suggests that LRIG3 can both oppose LRIG1's tumor suppressing function as well as act as a redundant pathway for LRIG1 function, possibly as a result of tissue- and subcellular localization- dependency or in the event of injury. These seemingly conflicting pieces of evidence only highlight the need for a better functional analysis of this protein both within specific tissues and in juxtaposition with other members of the LRIG family. And because we know that LRIG1 expression levels can be strongly correlated to quite a few cancer prognoses, it is reasonable to wonder if LRIG3-expression levels might also better indicate the outcomes of a broader range of cancer types. While this paper unfortunately does not provide these intriguing results, it does offer a better analysis of the localization of LRIG1 within the colonic crypt and, as a result, a better understanding of the possible localization of LRIG3 within the colonic crypt.

Background: Immunofluorescence

Antibodies, or immunoglobulins, are Y-shaped molecules produced by the immune system. They target molecules called antigens by specifically binding to a part

on the antigen called an epitope (Figure 7). These antigens can be foreign substance in the body.

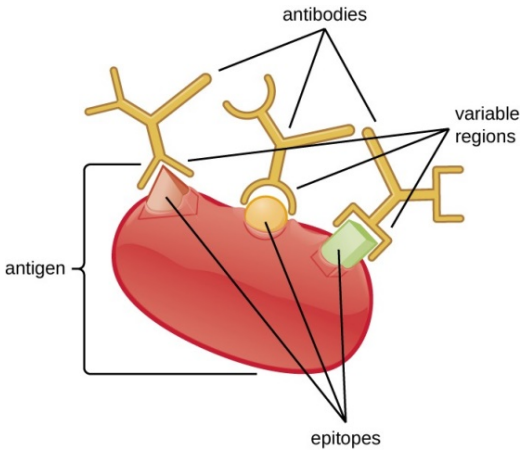

Figure 7: Antibody binding of antigen.

Unique patterns of amino acids in the variable regions of the antibodies allow for specific binding of the antibody to its epitope, which is expressed on the target antigen (https://courses.lumenlearning.com/microbiology/chapter/polyclonal-and-monoclonal-antibody-production/).

This highly specific antibody/antigen interaction is utilized in immunohistochemistry (IHC), which is a powerful technique for the identification of specific tissue components. Antibodies are created in animal models such that the variable region[26] is made to bind a desired cell component target. Therefore, when this antibody is washed over a tissue sample, the target cell component is bound. Identification of the bound cell component can be derived from immunofluorescence, or the attachment of fluorophores to the antibodies such that light microscopy can detect antibody localization.

[26] Variable region – located on the tips of the Y-shaped antibody, this region is structurally varied between each antibody such that many different antigens can be bound to many different antibodies.

Fluorophores are chemical compounds that can re-emit a colored light upon light excitation, and they are either directly or indirectly conjugated to antibodies.[27]

[27] Direct fluorophore conjugation has the fluorophore attached to the antibody that directly binds the cell component target. Indirect fluorophore conjugation is when the fluorophore is attached to an antibody that targets a different antibody that targets the actual cell component.

Hypothesis

Objectives of study

There are two objectives to this study:

1) We will evaluate the average topmost LRIG1-expressing cell within the colonic crypt.

2) We will evaluate the average total number of Ki67/LRIG1 co-positive cells in order to create a proliferative character of the colonic crypt.

These two objectives will be accomplished by comparing wild-type[28] (WT) mice with *Lrig3*$^{-/-}$ mice.

For the first objective, if LRIG1-expressing cells are observed on average to be at a higher cell position up the crypt wall in *Lrig3*$^{-/-}$ mice relative to WT mice, then we can say that LRIG1 has shifted up the crypt wall in the absence of *Lrig3* product. If LRIG1-expressing cells are observed on average to be in the same location between WT and *Lrig3*$^{-/-}$ mice, then we can say that absence of *Lrig3* product does not affect LRIG1 localization within the colonic crypt. Lastly, if LRIG1-expressing cells have moved down the crypt wall in *Lrig3*$^{-/-}$ mice compared to WT mice, we can say that LRIG1 has localized at a lower cell position within the colonic crypt in the absence of *Lrig3* product.

The second objective will be evaluated by counting the number of Ki67/LRIG1 co-positive cells within colonic crypts that are also analyzed for the first objective. Cellular Ki67 expression is correlated with proliferation of the cell, and if analyzed within a larger tissue structure can be correlated with that structure's proliferative index

[28] Wild-type – genetic designation given to indicate that a strain, gene, or characteristic is prevalent among organisms in natural conditions, and is distinct from an atypical mutant type.

as a whole. As such, the more Ki67/LRIG1 co-positive cells that are counted within a colonic crypt, the greater the proliferative index of that crypt; the fewer the number of Ki67/LRIG1 co-positive cells within a crypt, the lesser the proliferation of the tissue structure as a whole. Comparing the proliferative index of colonic crypts between WT and *Lrig3*$^{-/-}$ mice will allow us to analyze how proliferation of the crypts may change in the absence of *Lrig3*, and thus how *Lrig3* may function within the colonic crypts.

Overall, we can only speculate on the localization of LRIG3 protein within WT mice, although we know at the very least that *Lrig3* mRNA transcripts are found overlapping and just above the LRIG1-expressing cells found in the colonic crypt base. Therefore, in regards to the previously mentioned evidence of LRIG1 directly opposing LRIG3, we hypothesize that when *Lrig3* is not present, LRIG1 will be expressed at greater levels within the colonic crypts, and that the colonic crypt will be less proliferative as a whole due to the relatively quiescent nature of LRIG1.

Importance of study

Regulation of the colon is vital for homeostasis and health of the body as a whole. As such, dysregulation of the mechanistic pathways within this organ can lead to numerous, sometimes lethal pathologies. One such pathology is tumorigenesis, which arises from uncontrolled cell growth. The LRIG protein family has been studied in large part because of its interactions with biomolecules that are part of mechanistic pathways involved in cell growth. It has been discovered that LRIG1, for example, is a tumor suppressor in the colon, as studies have shown tumor formation in response to *Lrig1* deletion (Powell et al., 2014).

However, the other LRIG members are less well studied, and as such their function and localization in certain tissues is still fairly unknown. LRIG3's unknown function and localization within the colon is one such example. By understanding where LRIG1-expressing cells are found in the colonic crypt in absence of *Lrig3* products, we can better suggest where *Lrig3* products might be located in relation to LRIG1within the colonic crypt. As such, this study aims to elucidate simple interaction characteristics of LRIG1 and *Lrig3* products, such that future studies can build upon this data and better determine the role and localization of these LRIG members in proximity.

Results

Wild-type (WT) mice were compared against *Lrig3*$^{-/-}$ mice in order to analyze if LRIG1-expressing cells of the colonic crypt localized differently in the absence of *Lrig3* product. We hypothesized that, due to the functional opposition that LRIG1 presents against LRIG3, LRIG1-expressing cells would move up the colonic crypt in absence of LRIG3 interaction. Preparation of the colons of WT and *Lrig3*$^{-/-}$ mice was followed by immunofluorescence staining of particular cell components. These fluorescent components were viewed using light microscopy, and captured images of the stained colonic crypts were analyzed in order to determine any shifts in localization of LRIG1-expression. This was done by analyzing 150 colonic crypts in three, double-blinded[29]samples each of WT and *Lrig3*$^{-/-}$ mice, counting 1) the topmost +position of LRIG1-expressing cells, and 2) the total number of Ki67/LRIG1 co-positive cells found below the topmost LRIG1-expressing cell.

Preparation and immunofluorescence of colonic crypts

Three colons each from WT and *Lrig3*$^{-/-}$ mice were cleaned, fixed, and placed on slides in anticipation of immunofluorescence staining (see Methods). Each of the six mice samples produced twenty tissue samples for analysis. All tissue samples were stained for DAPI, Ki67, and LRIG1 (Figure 8).

[29] Double-blind (control) – when neither the main experimenter nor any involved colleagues know the particulars of a study's variable; used to prevent bias in a study

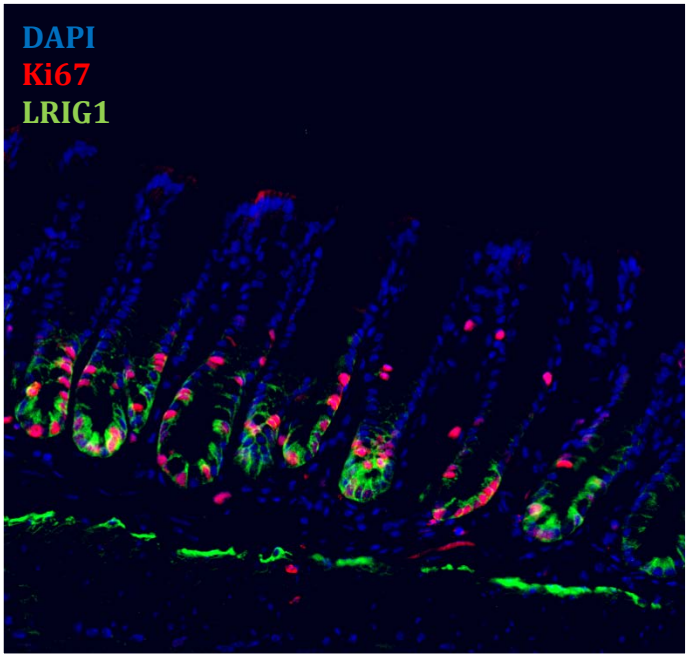

Figure 8: Colonic crypt immunofluorescence under light microscopy.

Light microscopy displays an image of fluorescently stained colonic crypts, which provides a simultaneous view of cell nuclei (blue), proliferating cells (red), and LRIG1-expressing cells (green).

Light microscopy of each of the three immunofluorescence stains revealed distinct colonic crypts with LRIG1-expresing cells concentrated around the base of the crypt. Proliferating cells – marked by Ki67 – were also observed within the crypt; the majority of these cells were located in the bottom half of the colonic crypt.

Counting cells of the colonic crypt

Each crypt was scored for two data points: 1) the +position of the topmost LRIG1-positive cell, and 2) the total number of Ki67/LRIG1 co-positive cells that were found below the topmost LRIG1-positive cell (Figure 9).

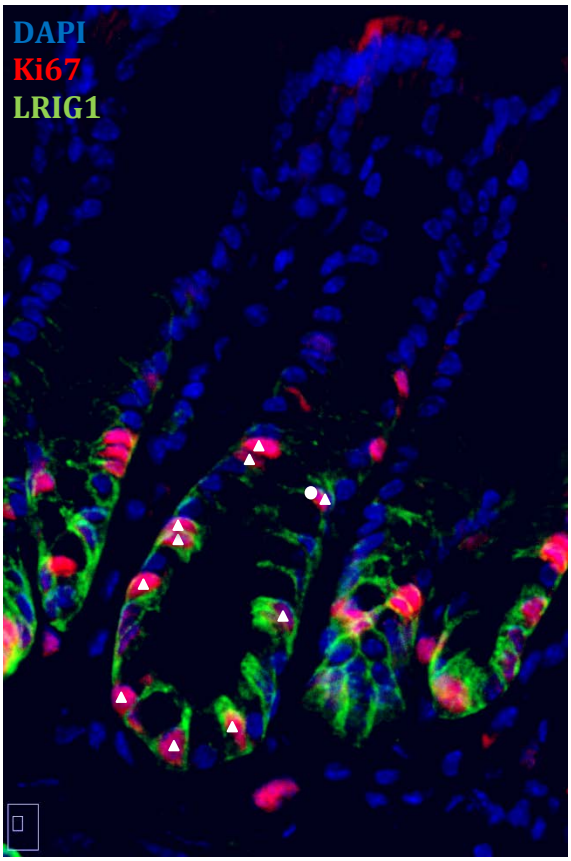

Figure 9: Counting LRIG1- and Ki67/LRIG1-expressing cells in the colonic crypt.

Two data points were collected for each crypt selected: 1) the position of the most lumenal LRIG1-positive cell (white circle), and 2) the total number of Ki67/LRIG1 co-positive cells found below the most lumenal LRIG1-positive cell (white triangles). The colonic crypt was fluorescently labeled for cell nuclei (blue), proliferating cells (red), and LRIG1-expressing cells (green).

The +position for LRIG1-expressing cells was found by first deciding the cell with position 0 (the most basal, or bottommost, cell of the crypt), and then counting up on either side of cell 0 until reaching what was determined to be the most lumenal LRIG1-expressing cell. This cell was designated a +position, and the data for that distinct crypt was recorded. Each sample had this data recorded for 50 distinct colonic crypts, and each of the 50 crypt's data was averaged (Figure 10).

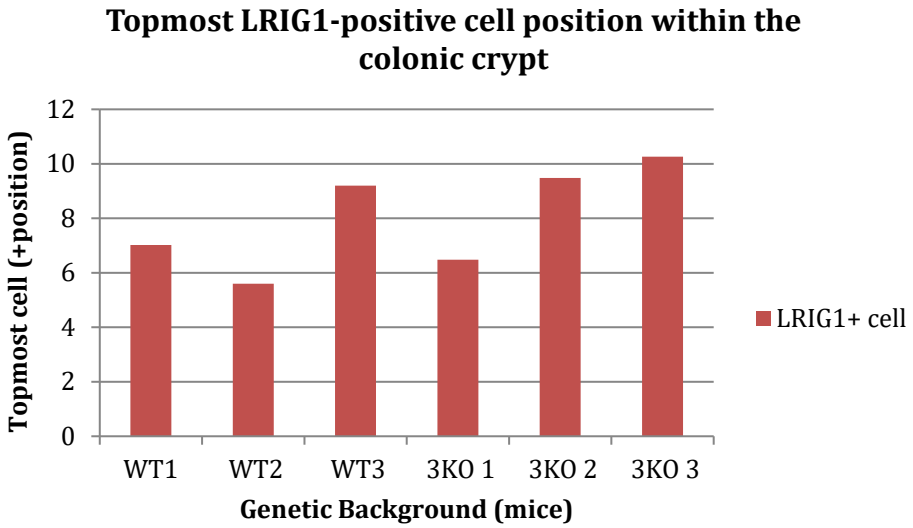

Figure 10: Analysis of topmost LRIG1-positive cells in the colonic crypts of wild-type and *Lrig3*$^{-/-}$ mice.

For each of the six mouse samples, 50 colonic crypts were analyzed for the +position of the most lumenal LRIG-positive cell. This data was visually compared between the wild-type (WT) and *Lrig3*$^{-/-}$ (3KO) colonic crypt samples.

Within the same crypt, the total number of Ki67/LRIG1 co-positive cells that were below the most lumenal LRIG1-expressing cell were counted and also recorded. Once again, each sample's data was averaged over 50 unique colonic crypts within the sample (Figure 11).

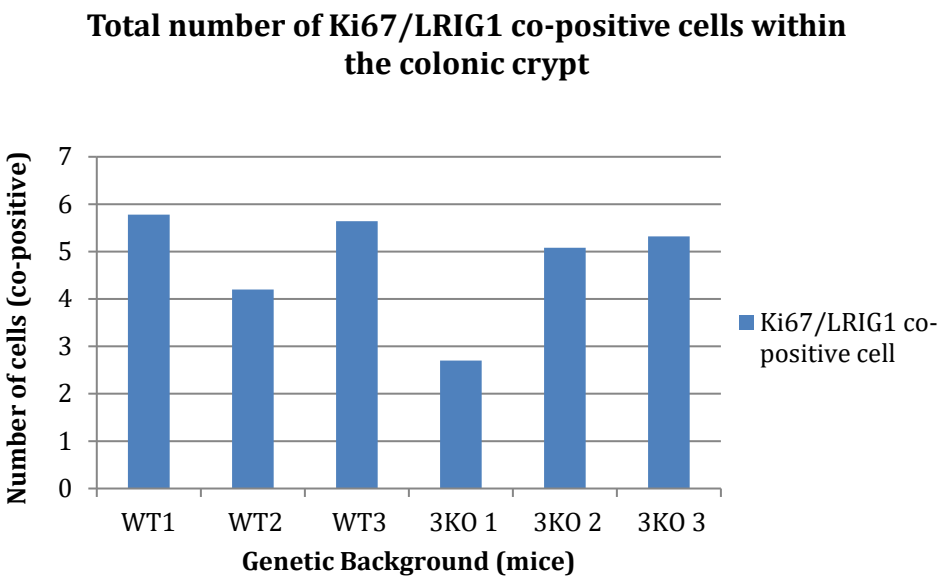

Figure 11: Analysis of Ki67/LRIG1 co-positive cells in the colonic crypts of wild-type and *Lrig3*$^{-/-}$ mice.

For each of the six mouse samples, 50 colonic crypts were analyzed for the total number of Ki67/LRIG1 co-positive cells. This data was visually compared between the wild-type (WT) and *Lrig3*$^{-/-}$ (3KO) colonic crypt samples.

The two, averaged data points for each of the six samples is displayed numerically below (Table 1).

	WT1	WT2	WT3	3KO 1	3KO 2	3KO 3
Total Ki67/LRIG1 co-positive cells	5.78	4.20	5.64	2.70	5.08	5.32
Topmost LRIG1-positive cell	7.02	5.60	9.20	6.48	9.48	10.26

Table 1: Data point averages between the three wild-type and three *Lrig3*$^{-/-}$ mice samples.

Each of the six samples was analyzed for two data points. The three wild-type (WT) samples were numerically compared to the three *Lrig3*$^{-/-}$ (3KO) samples.

Once each sample had both data points recorded for each of the 50 colonic crypts, the double blind control was removed and the samples were correlated as either WT or

Lrig3$^{-/-}$. Table 2 displays the results of averaging together the samples that were WT and *Lrig3*$^{-/-}$.

	WT	*Lrig3*$^{-/-}$	Change
Total Ki67/LRIG1 co-positive cells	5.21	4.37	-0.84
Topmost LRIG1-positive cell	7.34	8.74	+1.47

Table 2: Data point averages between wild-type and *Lrig3*$^{-/-}$ mice.

Each of the six samples was analyzed for two data points. The three wild-type (WT) samples were averaged and then numerically compared to the three *Lrig3*$^{-/-}$ samples that were also averaged.

The +position for the topmost LRIG1-expressing cell within the colonic crypt was different between the WT and *Lrig3*$^{-/-}$ mice, with the *Lrig3*$^{-/-}$ mice expressing LRIG1 approximately 1.47 cells higher up the crypt wall relative to WT (Figure 12).

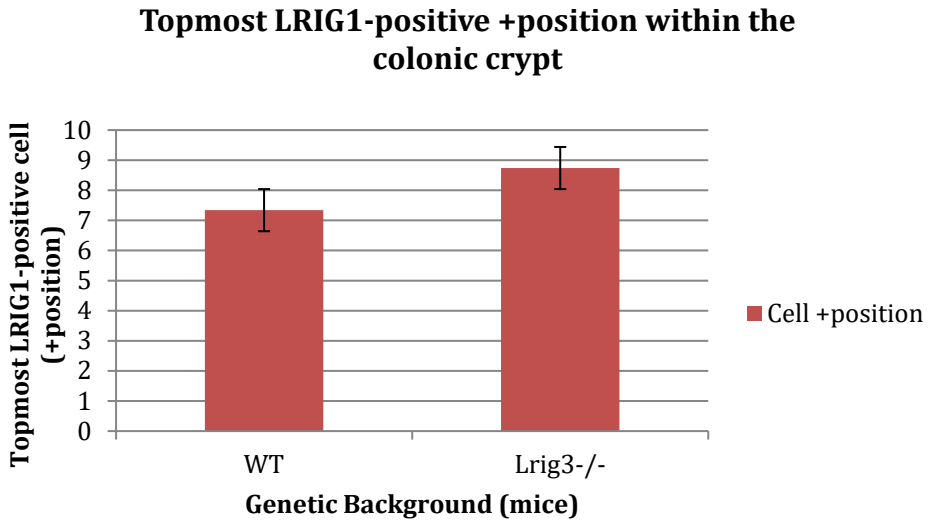

Figure 12: Comparison of the topmost +position of LRIG1-expressing cells in the colonic crypt between wild-type and *Lrig3*$^{-/-}$ mice.

For each of the two different genetic backgrounds, a total of 150 colonic crypts were analyzed and averaged for the topmost +position of LRIG1. An unpaired t-test between the two genetic groups determined that the difference is extremely statistically significant, with a two-tailed P value less than 0.0001 (see Methods). The standard deviation for wild-type (WT) was 2.59, while *Lrig3*$^{-/-}$ had a standard deviation of 3.02.

This difference is extremely statistically significant, indicating that the upward shift of LRIG1-expressing cells was not random, but the methodical result of the genetic difference between the two samples. In this case, we can assert that absence of *Lrig3* within the colonic crypt caused LRIG1 to be expressed approximately 1.47 cells higher up the crypt wall.

The total number of Ki67/LRIG1 co-positive cells within the colonic crypt also differed between the WT and *Lrig3*$^{-/-}$ mice (Figure 13).

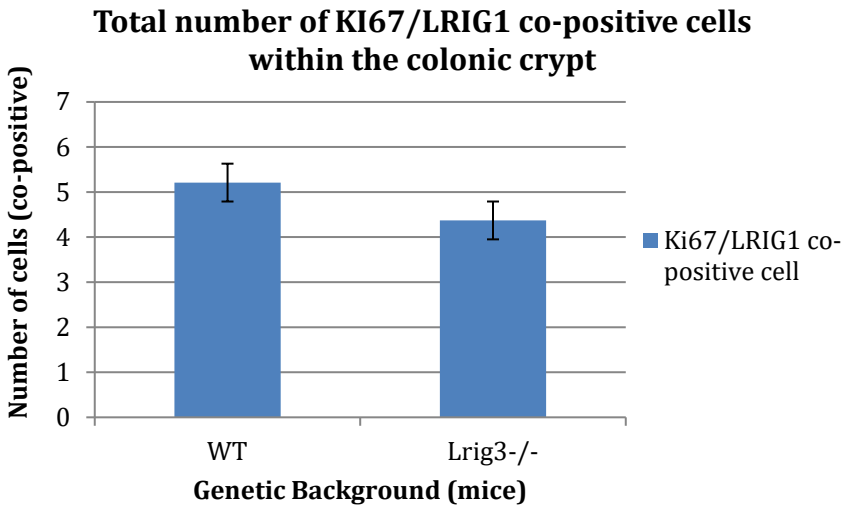

Figure 13: Comparison of the total number of Ki67/LRIG1 co-positive cells in the colonic crypt between wild-type and *Lrig3*$^{-/-}$ mice.

For each of the two different genetic backgrounds, a total of 150 colonic crypts were analyzed and averaged for the total number of Ki67/LRIG1 co-positive cells. An unpaired t-test between the two genetic groups determined that the difference is very statistically significant, with a two-tailed P value less than 0.0017 (see Methods). The standard deviation for both wild-type (WT) and *Lrig3*$^{-/-}$ mice was 2.30.

In this case, the *Lrig3*$^{-/-}$ mice had approximately 0.84 fewer Ki67/LRIG1 co-positive cells within the colonic crypt relative to WT mice. Again, the difference is statistically significant between the two groups of mice, providing evidence that the decrease in Ki67/LRIG1 co-positive cells seen in the *Lrig3*$^{-/-}$ mice was a direct result of the absence of *Lrig3*.

On average, LRIG1 was expressed approximately 1.47 cells higher up the colonic crypt wall in the *Lrig3*$^{-/-}$ versus the WT mice. However, the *Lrig3*$^{-/-}$ mice had an average of 0.84 fewer Ki67/LRIG1 co-positive cells in their colonic crypts relative to WT mice. Thus, in general, the *Lrig3*$^{-/-}$ colonic crypt had LRIG1-expressing cells higher up the crypt wall compared to WT, but had fewer cells that were both proliferating and expressing LRIG1 in total.

However, after further consideration, it was determined that the two data points described above should be re-analyzed to better capture the total statistical significance of our findings. As such, one-way analysis of variance (ANOVA) was used to test for statistical significance among the three WT samples and the three *Lrig3*$^{-/-}$ samples. Both WT and *Lrig3*$^{-/-}$ tests for each of the two data points determined that there was indeed statistical significance among the two genetic backgrounds, and as such signified that we could not determine if there was actual statistical significance between the WT and *Lrig3*$^{-/-}$ mice. These statistics are displayed for the topmost LRIG1-positive cell position in Figure 14:

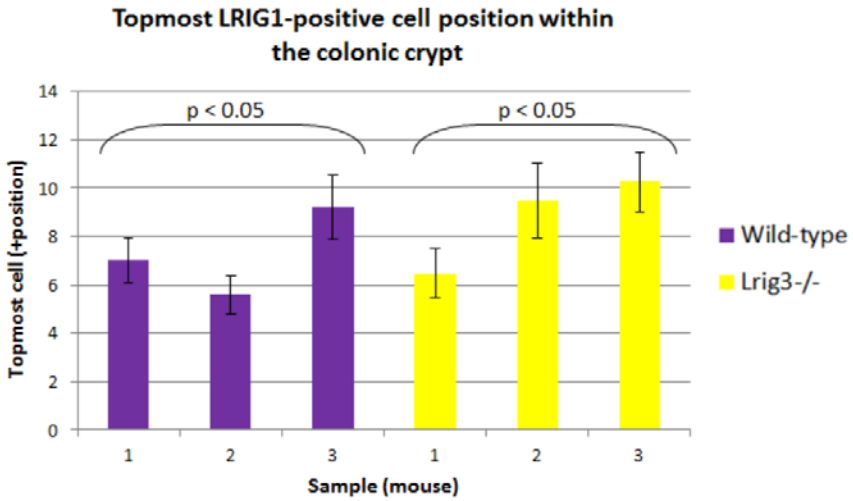

Figure 14: Comparison of topmost LRIG-positive cells in the colonic crypt between wild-type and *Lrig3*$^{-/-}$ mice.

The three samples of each genetic background were analyzed for statistical significance between the 50 colonic crypts counted for each sample. A one-way ANOVA test determined that there was statistical significance among both the wild-type (WT) and *Lrig3*$^{-/-}$ samples ($p < 0.05$). As such, statistical significance could not be determined between the WT and *Lrig3*$^{-/-}$ samples. Bars signify standard deviation for each sample.

Figure 15 displays the one-way ANOVA statistics for the total number of Ki67/LRIG1 co-positive cells within the colonic crypts:

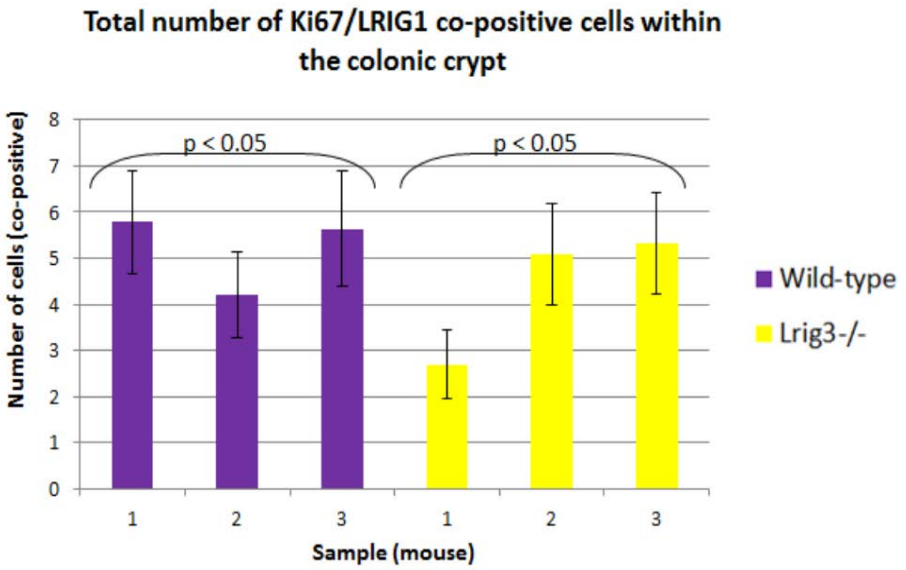

Figure 15: Comparison of Ki67/LRIG1 co-positive cells in the colonic crypt between wild-type and *Lrig3*$^{-/-}$ mice.

The three samples of each genetic background were analyzed for statistical significance between the 50 colonic crypts counted for each sample. A one-way ANOVA test determined that there was statistical significance among both the wild-type (WT) and *Lrig3*$^{-/-}$ samples ($p < 0.05$). As such, statistical significance could not be determined between the WT and *Lrig3*$^{-/-}$ samples. Standard deviation for each sample is signified by bars.

These new statistics suggest that for a more complete analysis of the colonic crypt differences between WT and *Lrig3*$^{-/-}$ mice, we should implement methods that better collect and quantify the data. One possibility would be to define a more rigid set of parameters for counting the two data points; for examples, the Ki67/LRIG1 co-positive cells could be counted in relation to the total cell height of the crypt, making the co-positive cells a percentage rather than a total number. This would better delineate that actual amount of proliferation within a crypt as it would take into account the variances of crypt height. Another was to determine statistical significance between the WT and

Lrig3$^{-/-}$ mice would be to simply count more overall samples. This would increase samples size and provide a more accurate and precise analysis of the two data points. Additionally, counting more colonic crypts for the samples already collected would be another way to improve accuracy and precision of the two data points.

Discussion

LRIG3 is a member of the leucine rich repeats and immunoglobulin-like domains (LRIG) protein family, but only limited research has been done to explore its functional role and localization within the body. Conversely, both LRIG1's function and localization is much more defined within the body; for example, LRIG1 is definitively known as a tumor suppressor in the colon. As such, LRIG1 provokes continued interest from the research community both for its application in cancer prognoses and its potential as a tumor therapeutic.

Due to the similar homology between LRIG1 and LRIG3, there has been speculation that LRIG3's function might possess characteristic that either oppose or support LRIG1 function, and indeed, supporting evidence for both of these possibilities has been discovered. However, LRIG3's localization and functionality within the colon is still largely undefined. This study analyzes both LRIG1 expression and the proliferation index within the colonic crypts of WT and *Lrig3*$^{-/-}$ mice in order to better understand the possible localization and functionality of colonic crypt *Lrig3*.

Results of most lumenal LRIG1-positive cell

The results of this study show that in the absence of *Lrig3* product, there is a distinct, upward shift in localization of LRIG1-expressing cells within the colonic crypt. Specifically, data analysis indicates that LRIG1 is expressed 1.47 cells higher up the crypt wall. Keeping in mind that the colonic crypt is only 19-35 cells tall, this already appears to be a relatively significant shift in position, and indeed, an unpaired t-test affirms this shift to be extremely statistically significant ($P < 0.0001$) (Sunter et al., 1979). However, further statistical analysis revealed that there is also statistical

significance among the three WT and the three *Lrig3*$^{-/-}$ samples. As such, the earlier data that suggested that LRIG1 localizes higher up the colonic crypt may not be accurate. In the interest of treating the earlier results as potentially accurate, we can put forth several suggestions as to why LRIG1 migrates upward in the absence of *Lrig3*, based on the conclusion that 1) loss of *Lrig3* directly affects LRIG1 localization in colonic crypt, and that 2) here is cross-talk between LRIG1 and *Lrig3*.

The first scenario is that *Lrig3* product suppresses the upward migration of LRIG1-expressing cells. This may be done by direct interaction between *Lrig3* mRNA or protein with LRIG1 protein, *Lrig1*mRNA, or even the *Lrig1* transcription or translation processes. *Lrig3* product may indirectly inhibit upward LRIG1 migration, as previous evidence has shown LRIG1 to degrade LRIG3, so it may be possible that without needing to invest time or energy to do so, LRIG1 can extend further up the crypt wall.

A second possibility is that there might be direct suppression of LRIG1 transcription by *Lrig3* products. As such, cells with a certain level of *Lrig3* expression might hamper the simultaneous production of LRIG1. Regardless of the exact mechanistic nuances, some form of cross-talk between LRIG1 and *Lrig3* product is the most likely reason for the earlier results this study found, as we potentially see a direct change in LRIG1 localization in the absence of *Lrig3*.

Results of Ki67/LRIG1 co-positive cell numbers

Protein Ki67 is expressed in cells that are dividing, and as such, proliferating stem- and TA-cells can be localized within a tissue sample by labeling for Ki67 presence. Evidence has been found that LRIG1-positive intestinal stem cells are relatively inactive in terms of their rate of division, suggesting that LRIG1-expressing cells may be generally less likely to divide and therefore less likely to express Ki67. Therefore, the very statistically significant ($P < 0.0017$) 0.84 cell decrease in Ki67 and LRIG1 co-positive cells in the absence of *Lrig3* product is a reasonable observation, as the increase in LRIG1 may restrict a more robust rate of cell cycle division within the colonic crypt and thus a lack of Ki67. While the most recent statistics call this observation into question, we offer a discussion here that is related to the earlier statistics that found there was a noticeable change in proliferation index between WT and *Lrig3*$^{-/-}$ colonic crypts. As such, we can suggest that *Lrig3* product might either directly or indirectly encourage the growth and division of cells.

There is further support for the observed decrease of Ki67 within the colonic crypts when one considers that LRIG1 suppresses cell growth via the negative regulation of EGFRs. In other words, increased LRIG1 expression decreases cell growth and thus Ki67 presence. Moreover, LRIG3 has been observed to increase growth factor receptors in certain tissues and so increase the rate of cell division; thus, absence of *Lrig3* product would also contribute to an overall decrease in the rate of cell division. Therefore, both the increase in LRIG1-expressing cells and the absence of *Lrig3* product was likely to contribute to the 0.84 cell decrease in Ki67/LRIG1 co-positive cells seen within the colonic crypts of *Lrig3*$^{-/-}$ mice.

Due to LRIG1's role in the homeostasis of cell growth, LRIG1 is a protein of interest in studying tumor formation; as such, LRIG3 might also be a protein of interest due to evidence of its homology and interaction with LRIG1 in certain tissues. This study aims to contribute to the possible characterization of colonic crypt LRIG3 through analysis of both colonic crypt LRIG1 and proliferation index, such that future researchers might be able to move one step closer in fully elucidating LRIG3 functionality and localization.

Future Directions

Colitis is an inflammation of the colon that results in bloody stool and diarrhea, and can be induced in mice through addition of dextrane sulfate sodium (DSS) to a mouse's drinking water. Comparison of phenotypes between WT and genetically modified mice can reveal protective, deleterious, or neutral effects as a result of the genetic modification. For example, a DSS-treated mouse that has been genetically modified to not possess any LRIG1 (*Lrig1*$^{-/-}$) has comparatively more blood in their stools and a more inflamed colon as viewed by cross section in relation to WT; this suggests LRIG1 has a protective effect for the colon against colitis (Powell et al., 2014).

A future study could analyze the protective, deleterious, or neutral effect of LRIG3 in the colon via DSS-induced colitis treatment between WT and *Lrig3*$^{-/-}$ mice. This study would rely upon evaluation of behavioral phenotype and examination of tissue cross sections of the colon in mice with DSS-induced colitis. If *Lrig3*$^{-/-}$ mice displayed improved symptoms of colitis relative to WT mice, then LRIG3 would be considered to have a deleterious effect against colitis. Conversely, if *Lrig3*$^{-/-}$ mice present with worse symptoms of colitis in comparison to WT mice, than LRIG3 would

be considered to have a protective effect against colitis. Lastly, if *Lrig3*$^{-/-}$ mice demonstrate similar symptoms as WT mice, then LRIG3 would be characterized as having a neutral effect on colitis. Although the exact biochemical basis for the observed effects would not be determined in this study, the results would better elucidate the functionality of *Lrig3* product within the colon, ideally helping future researchers decide how to approach future mechanistic experimentation of LRIG3.

Methods

Colon preparation

Wild type (WT) and *Lrig3*$^{-/-}$ mice were sacrificed 12 weeks after birth. The colon was washed with 1X phosphate-buffered saline (PBS) solution and fixed flat with the lumenal side facing up on a wax surface with 4% paraformaldehyde and PBS buffer. The fixed colon was processed in 30% sucrose, after which the processed tissue samples were embedded in optimum cutting temperature (O.C.T) 'frozen' blocks on dry ice. The frozen blocks were stored at -80 °C. The blocks were then sectioned on a cryostat such that two, 5µm-thick tissue slices were placed on each glass slide. Each of the six mouse samples had a total of ten slides, with a total of twenty tissue sections for each sample and a total of sixty sections for each of WT and *Lrig3*$^{-/-}$ mice.

Immunofluorescence staining

The frozen, plated tissue sections were removed from -80 °C storage, air-dried for 15 minutes, and passed through three, 3-minute washes of 1X PBS. A hydrophobic pen was used to outline the tissue samples on the slides, after which the samples were incubated in PBS blocking buffer for 30 minutes in a moistened environment. Primary antibodies consisting of a 1:200 directly conjugated rat anti-KI67 PE antibody (eBioscience, catalog# 12-5698-82) and a 1:500 goat anti-LRIG1 antibody (Cosmo Bio, catalog# 25503-96, lot# 610G0675) were added to the samples and left overnight at room temperature.

The samples were again washed three times for three minutes each in 1X PBS. Secondary antibodies of 1:500 donkey anti-goat 647 (Jackson ImmunoResearch,

catalog# 705-605-147, lot# 121221) were added to each sample and allowed to incubate for one hour. The slides were then washed for three minutes in 1X PBS, then five minutes in 1X PBS+DAPI (1:10,000), then again in 1X PBS. Approximately 4 drops of n-propyl gallate was added to each slide, covered with a coverslip, and stored at 4 °C.

Crypt imaging and position counting

The tissue samples were placed under a fluorescent microscope; crypt morphology and biochemical makeup was viewed via the proliferation marker Ki67 (red), stem cell marker LRIG1 (green), and the cell nucleus (DAPI, blue). Each colored stain was its own 'channel'; a merged channel was the overlap of all three colored immunostains into one composite image as seen in Figure 8.

Each morphologically acceptable colonic crypt was scored on for: 1) the topmost +position of LRIG1, and 2) the total number of Ki67 and LRIG1 co-positive cells within the crypt but below the topmost LRIG1-positive cell. These data were analyzed such that each sample was averaged for topmost +position of LRIG1 and Ki67/LRIG1 co-positive cells. Each of the six samples (three WT and three *Lrig3*$^{-/-}$) were double blinded while counting occurred; after completion of counting, the double blind was removed and analysis was done with knowledge of which sample was WT and which was *Lrig3*$^{-/-}$.

Statistical significance

The online tool GraphPad QuickCalcs (https://www.graphpad.com/quickcalcs/ttest1.cfm) was used to calculate the unpaired t-test results shown in Figure 12 and Figure 13. The two data points for each of *Lrig3*$^{-/-}$ and WT's 150 colonic crypts were statistically analyzed for P value ($p < 0.05$), statistical significance, and standard

deviation via this program. The online tool One-Way ANOVA Calculator (http://www.socscistatistics.com/tests/anova/default2.Aspx) was used to calculate the one-way ANOVA statistics for Figure 14 and Figure 15. The two data points for the three WT and three *Lrig3*$^{-/-}$ samples were analyzed for P value ($p < 0.05$) and standard deviation of each sample.

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